

## PATENT COOPERATION TREATY

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## **NOTIFICATION OF ELECTION**

(PCT Rule 61.2)

|   |  |
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| <p><b>The International Bureau of WIPO</b><br/> <b>34, chemin des Colombettes</b><br/> <b>1211 Geneva 20, Switzerland</b></p> <p>Facsimile No.: (41-22) 740.14.35</p> | <p><b>Authorized officer</b></p> <p><b>S. Baharlou</b></p> <p>Telephone No.: (41-22) 338.83.38</p> |
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09/403897REC'D 12 AUG 1999  
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## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

|  |   |  |
|--|---|--|
| Applicant's or agent's file reference<br><br>Y/97-30 PCT   | <b>FOR FURTHER ACTION</b> See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416) |  |
| International application No.<br><br>PCT/IL98/00196  | International filing date (day/month/year)<br><br>26/04/1998  | Priority date (day/month/year)<br><br>29/04/1997 |
| International Patent Classification (IPC) or national classification and IPC<br><br>A61K38/33  |   |  |
| Applicant<br><br>YEDA RESEARCH AND DEVELOPMENT CO. LTD. et al.   |   |  |
| <p>1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.</p> <p>2. This REPORT consists of a total of 5 sheets, including this cover sheet.</p> <p><input type="checkbox"/> This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).</p> <p>These annexes consist of a total of sheets.</p>  |   |  |
| <p>3. This report contains indications relating to the following items:</p> <ul style="list-style-type: none"> <li>I    <input checked="" type="checkbox"/> Basis of the report</li> <li>II    <input type="checkbox"/> Priority</li> <li>III    <input type="checkbox"/> Non-establishment of opinion with regard to novelty, inventive step and industrial applicability</li> <li>IV    <input type="checkbox"/> Lack of unity of invention</li> <li>V    <input checked="" type="checkbox"/> Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement</li> <li>VI    <input type="checkbox"/> Certain documents cited</li> <li>VII    <input type="checkbox"/> Certain defects in the international application</li> <li>VIII    <input type="checkbox"/> Certain observations on the international application</li> </ul> |   |  |

|  |  |
|--|--|
| Date of submission of the demand<br><br>06/11/1998   | Date of completion of this report<br><br>11.12.98                        |
| Name and mailing address of the international preliminary examining authority:<br><br><br>European Patent Office<br>D-80298 Munich<br>Tel. (+49-89) 2399-0 Tx: 523656 epmu d<br>Fax: (+49-89) 2399-4465 | Authorized officer<br><br>Stoltner, A<br><br>Telephone No. (+49-89) 2399 |



**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/IL98/00196

**I. Basis of the report**

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

**Description, pages:**

1-23                   as originally filed

**Claims, No.:**

1-27                   as originally filed

**Drawings, sheets:**

1/9-9/9               as originally filed

2. The amendments have resulted in the cancellation of:

the description,      pages:  
 the claims,           Nos.:  
 the drawings,          sheets:

3.  This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/IL98/00196

**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

**1. Statement**

|                               |  |
|-------------------------------|--|
| Novelty (N)                   | Yes: Claims 1-18   |
|                               | No: Claims 19-27   |
| Inventive step (IS)           | Yes: Claims  |
|                               | No: Claims 1-27  |
| Industrial applicability (IA) | Yes: Claims 1-27 (except some contracting states within the PCT) |
|                               | No: Claims   |

**2. Citations and explanations**

**see separate sheet**

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/IL98/00196

**ad section V:**

- 1). For the assessment of the present claims 1-10 and 27 on the question whether they are industrially applicable, no unified criteria exist in the PCT. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.
- 2). The present application refers to leptin and to its useful application in the field of oncology. Claim 1 is directed to the use of leptin and the derivatives thereof for the inhibition of tumor cell proliferation.
- 3). The following documents have been considered in relation to the present application:
  - D1, 5th. Ann. Conf. Int. Cytokine Soc. South Lake Tahoe, 9(11), , 9-13/11/97, Nevada USA, pp. 953, Rubinstein M. et al., an intermediate document, discloses the inhibition of growth-factor induced cell proliferation by leptin (cf. the whole abstract). Moreover, D1 points at the possible treatment of breast tumor cells and tumors originating from other tissues due to its cell proliferation inhibiting effects. However, D1 is dated 11/97 and has been published after the priority date of the present application. As the use of leptin for both the inhibition of the growth factor induced cell proliferation as well as the inhibition of breast cancer are disclosed in the priority document (Israel application No. 120733), D1 cannot prejudice the present subject-matter.
  - D2, Endocrinology, 138(2), 2/97, pp. 847-850, Zachow R.J. et al. reports the dose-dependent inhibition of IGF-1 increase by leptin in FSH-stimulated estradiol-17 $\beta$  production (cf. abstract). D2 rather concerns mechanisms in female infertility mentioning IGF-1 as having sensitizing effects in dominant follicles which may be inhibited by leptin. This has no relevance to the present subject-matter.

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/IL98/00196

D3, WO-A-9 727 286, refers to the use of the ob-gene product for stimulating the hematopoietic development (cf. abstract). D3 also seems to concern the treatment of tumor cells by administration of leptin: In page 33, lines 25-28, D3 apparently suggests the use of leptin for suppressing tumor growth which also includes tumor cell proliferation.

- 4). With regard to the disclosure of D2 the use of an active agent as depicted in claim 1 for the manufacture of a medicament for **inhibiting tumor cell proliferation** is nowhere disclosed nor suggested. However, D3, although silent on the explicit therapeutical use of leptin and its derivatives in the inhibition of tumor cell proliferation, strongly points out its possible therapeutical use for suppressing tumor growth. As consequence the subject-matter of the present claims 1-18 does not appear inventive pursuant to Art. 33(3) PCT.
- 5). Claims 19-26 refer to a pharmaceutical composition comprising the agents depicted in claim 1. From the cited documents D2 and D3 compositions comprising leptin and congeners thereof are well known in the prior art. The subject-matter of claims 19-26 as such does not fulfil the requirements of Arts. 33(2) and 33(3) PCT.

## PATENT COOPERATION TREATY

09/403897

PCT

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

|   |   |  |
|---|---|--|
| Applicant's or agent's file reference<br><b>Y/97-30 PCT</b>       | <b>FOR FURTHER ACTION</b> see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below. |  |
| International application No.<br><b>PCT/IL 98/ 00196</b>          | International filing date (day/month/year)<br><b>26/04/1998</b>   | (Earliest) Priority Date (day/month/year)<br><b>29/04/1997</b> |
| Applicant<br><b>YEDA RESEARCH AND DEVELOPMENT CO. LTD. et al.</b> |   |  |

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

It is also accompanied by a copy of each priorart document cited in this report.

1.  Certain claims were found unsearchable(see Box I).
2.  Unity of invention is lacking(see Box II).
3.  The international application contains disclosure of a nucleotide and/or amino acid sequence listing and the international search was carried out on the basis of the sequence listing
  - filed with the international application.
  - furnished by the applicant separately from the international application,
    - but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.
  - Transcribed by this Authority
4. With regard to the title,  the text is approved as submitted by the applicant
  the text has been established by this Authority to read as follows:
5. With regard to the abstract,
  - the text is approved as submitted by the applicant
  - the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this International Search Report, submit comments to this Authority.
6. The figure of the drawings to be published with the abstract is:
 

Figure No. \_\_\_\_\_

  - as suggested by the applicant.
  - because the applicant failed to suggest a figure.
  - because this figure better characterizes the invention.

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/IL 98/00196

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 A61K38/33

According to International Patent Classification(IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
|------------|--|-----------------------|
| P, X       | RUBINSTEIN M., BARKAN D. ET AL.: "Leptin inhibits growth factor induced cell proliferation"<br>FIFTH ANN. CONFERENCE OF THE INT. CYTOKINE SOC. LAKE TAHOE,<br>vol. cytokine 9, no. 11, 9 - 13 November<br>1997, NEVADA, USA,<br>page 953 XP002074549<br>*cf. abstract*<br>---<br>-/- | 1-27                  |



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

## ° Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

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Date of the actual completion of the international search

10 September 1998

Date of mailing of the international search report

18/09/1998

Name and mailing address of the ISA

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Stoltner, A

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/IL 98/00196

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document, with indication, where appropriate, of the relevant passages  | Relevant to claim No. |
|----------|---|-----------------------|
| Y        | ZACHOW R.J. , MAGOFFIN D.A.: "Direct intraovarian effects of leptin: Impairment of the synergistic action of insulin-like growth factor I on follicle-stimulating hormone dependent estradiol-17. $\beta$ production by rat ovarian granulosa cells" ENDOCRINOLOGY, vol. 138, no. 2, February 1997, pages 847-850, XP002074550<br>*cf. abstract*<br>--- | 1-27                  |
| P, Y     | WO 97 27286 A (PROGENITOR, INC.) 31 July 1997<br>*cf. page 5, lines 23-28, page 6, lines 21-37, page 9, lines 5-10, page 33, lines 25-31*<br>-----  | 1-27                  |

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International Application No

PCT/IL 98/00196

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|--|------------------|-------------------------|------------------|
| WO 9727286 A                           | 31-07-1997       | AU 1831197 A            | 20-08-1997       |

PATENT COOPERATION TREATY

From the  
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY



PCT

To:

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Inter-Lab Ltd.  
Science-based Industrial Park  
Kiryat Weizmann  
76110 Ness-Ziona  
ISRAEL

NOTIFICATION OF TRANSMITTAL OF  
THE INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT

(PCT Rule 71.1)

Date of mailing  
(day/month/year)

15. 08. 99

Applicant's or agent's file reference  
Y/97-30 PCT

IMPORTANT NOTIFICATION

International application No.  
PCT/IL98/00196

International filing date (day/month/year)  
26/04/1998

Priority date (day/month/year)  
29/04/1997

Applicant

YEDA RESEARCH AND DEVELOPMENT CO. LTD. et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.
2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.
3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

**4. REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/



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PCT

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

|  |                          |                          |  |
|--|--------------------------|--------------------------|--|
| (51) International Patent Classification <sup>6</sup> :<br><br>A61K 38/33  |                          | A1                       | (11) International Publication Number: WO 98/48831<br><br>(43) International Publication Date: 5 November 1998 (05.11.98)  |
| (21) International Application Number:   | PCT/IL98/00196           |                          | (81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). |
| (22) International Filing Date:  | 26 April 1998 (26.04.98) |                          |  |
| (30) Priority Data:  | 120733                   | 29 April 1997 (29.04.97) | IL   |
| (71) Applicant ( <i>for all designated States except US</i> ): YEDA RESEARCH AND DEVELOPMENT CO. LTD. [IL/IL]; Weizmann Institute of Science, P.O. Box 95, 76100 Rehovot (IL).   |                          |                          | <b>Published</b><br><i>With international search report.</i><br><i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>  |
| (72) Inventors; and<br>(75) Inventors/Applicants ( <i>for US only</i> ): BARKAN, Dalit [IL/IL]; Vilkomitch Street 22a, 76448 Rehovot (IL). COHEN, Batya [IL/IL]; Bloch Street 48, 64681 Tel Aviv (IL). RUBINSTEIN, Menachem [IL/IL]; Hatomer Street 16, 54042 Givat Shmuel (IL). |                          |                          |  |
| (74) Agent: EINAV, Henry; Inter-Lab Ltd., Science-based Industrial Park, Kiryat Weizmann, 76110 Ness-Ziona (IL).   |                          |                          |  |
| (54) Title: LEPTIN AS AN INHIBITOR OF TUMOR CELL PROLIFERATION   |                          |                          |  |
| (57) Abstract  |                          |                          |  |
| The use of leptin and leptin-related molecules in oncology is disclosed.   |                          |                          |  |

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

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## LEPTIN AS AN INHIBITOR OF TUMOR CELL PROLIFERATION

### FIELD OF THE INVENTION

5 The present invention relates to leptin, a cytokine produced by adipocytes and affecting a variety of cells and tissues. More particularly, this invention relates to novel applications of leptin in the field of oncology.

### BACKGROUND OF THE INVENTION

10 Leptin, an adipocyte-derived cytokine that regulates body weight, was identified by positional cloning of the murine *ob* gene (Zhang et al., 1994) and was shown to affect both food intake and thermogenesis (Campfield et al., 1995; Collins et al., 1996; Halaas et al., 1995; Pelleymounter et al., 1995; Weigle et al., 1995). High affinity leptin-binding sites were located in the choroid plexus; expression cloning of cDNA from this tissue provided the leptin  
15 receptor (OB-R) (Tartaglia et al., 1995). The known activities of leptin are mediated through its receptor in the hypothalamus. Leptin receptors are expressed, nonetheless, in additional organs, notably the kidney, lung and liver (Cioffi et al., 1996; Lee et al., 1996; Tartaglia et al., 1995). Furthermore, a different repertoire of leptin receptor-splice variants, differing in their cytoplasmic domain, is expressed in a tissue-specific manner in the mouse (Lee et al., 1996).  
20 Therefore, in addition to control of food intake and body heat, leptin may exert other physiological functions.

Although leptin is produced by adipocytes, the recent finding of a correlation between excess fat and high levels of leptin in the serum was in contrast with the notion that leptin reduces food intake and body weight (Considine et al., 1996; Frederich et al., 1995; Lonnqvist et al., 1995; Maffei et al., 1995). This correlation, and the well established linkage between obesity and insulin resistance (Felber and Golay, 1995), suggested that leptin may modulate insulin-regulated responses. Indeed, it was recently reported that leptin reduced significantly the basal and insulin-induced tyrosine-phosphorylation of the insulin receptor substrate-1 (IRS-1). This effect of leptin on IRS-1 phosphorylation was specific, since tyrosine-phosphorylation of the insulin receptor (IR)  $\beta$ -chain was not reduced (Cohen et al., 1996).

Tyrosine-phosphorylation of IRS-1 by the IR kinase is a key step in the insulin receptor signaling cascade, leading to many of the known insulin activities (Araki et al., 1994; Cheatham and Kahn, 1995; Myers et al., 1994; Myers et al., 1994; Myers and White, 1993; Rose et al., 1994; Tamemoto et al., 1994; White and Kahn, 1994). Downstream signaling of 5 IRS-1 is mediated by several associated proteins, one of which is the Growth-factor Receptor-associated Binding protein-2 (GRB2) (Cheatham and Kahn, 1995).

The insulin receptor (IR) is regarded as a metabolic receptor, mediating the effects of insulin on glucose homeostasis. As such it is expressed on terminally differentiated tissues such as adipose tissue, liver and muscle. However, many studies have shown that IR is a 10 potent mitogenic receptor in vitro and in vivo when expressed in tumor cells. For instance, functional IRs were identified in several breast cancer cell lines, as determined by tyrosine-phosphorylation of the IR in response to insulin treatment. Furthermore, the IR mediates a mitogenic response in these cells, as determined by [<sup>3</sup>H]thymidine incorporation (Milazzo et al., 1997; Millazzo et al., 1992).

15 Heretofore there has not been described the use of leptin in the field of oncology, in general; and in particular, leptin has not been described as being useful for inhibiting the proliferation of cells, especially proliferating cancer cells.

#### SUMMARY OF THE INVENTION

20 It is an object of the present invention to provide for the use of leptin as an inhibitor of cell proliferation, for example, to use leptin as an inhibitor of the proliferation of cancer cells.

It is another aim of the invention to provide for the use of leptin alone or in combination with other therapeutic agents for the treatment of various malignancies.

25 It is a further object of the present invention to provide for the use of leptin, leptin fusion proteins, leptin muteins, leptin receptor agonists, active fragments or fractions of any one thereof, active analogs or derivatives of any one thereof, salts of any one thereof, and mixtures of any thereof, for the treatment of various malignancies.

It is yet another object of the present invention to provide pharmaceutical 30 compositions containing one or more of the above leptin, leptin fusion proteins, leptin muteins, leptin receptor agonists, active fragments or fractions of any one thereof, active

analogs or derivatives of any one thereof, and salts of any one thereof, for the treatment of various malignancies.

Other objects of the present invention will be set forth herein below or will be readily apparent from the following disclosure.

5       The present invention provides the use of leptin as an inhibitor of cell proliferation. Leptin may be useful, either alone or in combination with other therapeutic agents or approaches, for the treatment of various malignancies. A preferred embodiment of the invention is the use of leptin for the inhibition of human breast carcinoma cell proliferation. The proliferation of many types of tumor cells is increased in the presence of various growth  
10      factors such as insulin and IGF-I. The growth stimulatory effect of insulin and IGF-I on cells is mediated, at least in part, via the IRS-1/GRB2 pathway (Myers et al., 1993). This pathway is inhibited by leptin. Furthermore, IRS-1 is a substrate of receptor kinases of additional growth factors and cytokines, including IL-4 and IL-9 (Pernis et al., 1995; Yin et al., 1995; Yin et al., 1994). Therefore, leptin may inhibit the mitogenic responses of some or all of the  
15      aforementioned growth factors and cytokines, as well as other growth factors, thereby inhibiting the proliferation of a variety of tumor cells. Examples provided include inhibition of the IGF-I-induced proliferation and insulin-induced proliferation of the human breast cancer cell lines T-47D and MCF7. The present invention also provides the use of leptin, leptin fusion proteins, leptin muteins, leptin receptor agonists, or active fragments or fractions of  
20      any one thereof, and salts of any one thereof as well as pharmaceutical compositions containing leptin, leptin fusion proteins, leptin muteins, leptin receptor agonists, active fragments or fractions of any one thereof, or salts of any one thereof for the treatment of various malignancies.

More specifically, the present invention provides the use of an active agent selected  
25      from the group consisting of leptin, leptin fusion proteins, leptin muteins, leptin receptor agonists, active fragments or fractions of any one thereof, active analogs or derivatives of any one thereof, salts of any one thereof, and mixtures of any thereof, as an inhibitor of tumor cell proliferation.

Embodiments of the above aspect of the present invention include :

30           (i) the use of the above active agent as an inhibitor of cell proliferation for the treatment of malignancies in mammals.

(ii) the use of the above active agent as an inhibitor of growth-factor dependent tumors.

(iii) the use of the above active agent as an inhibitor of human breast carcinoma cell proliferation.

5 (iv) the use of the above active agent for the treatment of human breast carcinomas.

(v) the use of the above active agent as an inhibitor of the growth stimulatory effect of insulin and IGF-I on tumor cells, as mediated, at least partially, by the insulin receptor substrate-1(IRS-1)/growth-factor receptor-associated binding protein-2 (GRB2) 10 pathway.

(vi) the use of the above active agent as an inhibitor of the mitogenic responses in tumor cells to one or more receptor kinases, growth factors and cytokines of the group consisting of IL-4 and IL-9, for all of which IRS-1 is a substrate, for the treatment of tumors.

15 (vii) the use of the above active agent as an inhibitor of basal, IGF-I-induced and insulin-induced tumor cell proliferation for the treatment of human breast cancers.

(viii) the use of the above active agent wherein said active ingredient is leptin, and said leptin is used as said inhibitor or for said treatment.

Likewise, the present invention also provides for an active agent selected from the group consisting of leptin, leptin fusion proteins, leptin muteins, leptin receptor agonists, 20 active fragments or fractions of any one thereof, active analogs or derivatives of any one thereof, salts of any one thereof, and mixtures of any thereof, for use in the preparation of a medicament for the inhibition of tumor cell proliferation.

Embodiments of this aspect of the invention include :

25 (i) an active agent as above for use in the preparation of a medicament for the treatment of malignancies in mammals.

(ii) an active agent as above for use in the preparation of a medicament for the inhibition of growth-factor-dependent tumors.

(iii) an active agent as above for use in the preparation of a medicament for the inhibition of human breast carcinoma cell proliferation.

30 (iv) an active agent as above for use in the preparation of a medicament for the treatment of human breast carcinomas.

(v) an active agent as above for use in the preparation of a medicament for the inhibition of the growth stimulatory effect of IGF-I and insulin on tumor cells, as mediated, at least partially, by the IRS-1/GRB2 pathway.

5 (vi) an active agent as above for use in the preparation of a medicament for the inhibition of the mitogenic responses in tumor cells to one or more receptor kinases, growth factors and cytokines of the group consisting of IGF-I, IL-4 and IL-9, for all of which IRS-1 is a substrate, for the treatment of tumors.

10 (vii) an active agent as above for use in the preparation of a medicament for the inhibition of basal, IGF-I-induced and insulin-induced tumor cell proliferation, for the treatment of human breast cancers.

(viii) an active agent as above wherein said active agent is leptin, and said leptin is used for the preparation of said medicament.

15 Similarly, in another aspect, the present invention provides a pharmaceutical composition comprising as active ingredient an active agent as noted above and a pharmaceutically acceptable carrier, diluent or excipient, for the inhibition of tumor cell proliferation.

Embodiments of this aspect of the invention include :

(i) a pharmaceutical composition for the treatment of malignancies in mammals.

20 (ii) a pharmaceutical composition for the inhibition of growth-factor-dependent tumors.

(iii) a pharmaceutical composition for the inhibition of human breast carcinoma cell proliferation and thereby for the treatment of human breast carcinoma.

25 (iv) a pharmaceutical composition for the inhibition of the growth stimulatory effect of IGF-I and insulin on tumor cells, as mediated, at least partially, by the IRS-1/GRB2 pathway.

30 (v) a pharmaceutical composition for the inhibition of mitogenic responses in tumor cells to one or more receptor kinases, growth factors and cytokines of the group consisting of IL-4 and IL-9, for all of which IRS-1 is a substrate, and thereby for the treatment of tumors.

(vi) a pharmaceutical composition for the inhibition of basal, IGF-I-induced and insulin-induced tumor cell proliferation and thereby for the treatment of human breast cancers.

(vii) a pharmaceutical composition wherein said active ingredient is leptin.

5 The present invention also provides for a method for treating tumors in mammals or for inhibiting tumor cell proliferation in mammals comprising administering to a patient a pharmaceutical composition according to the invention as noted above in a suitable dosage form and by a suitable route of administration. Such dosage forms and routes of administration are usually determined by the professional practitioners following their  
10 examination of the patient.

Other aspects and embodiments of the present invention are set forth or will be readily apparent from the following detailed description of the invention.

#### DESCRIPTION OF THE FIGURES

15 Figure 1 shows the dependence of T-47D cell proliferation on insulin as determined by MTT staining.

Figure 2 shows the dependence of T-47D cell proliferation on IGF-I as determined by MTT staining.

20 Figure 3 shows the inhibition of insulin-induced T-47D cell proliferation in 10% fetal bovine serum (FBS) by murine leptin as determined by MTT staining.

25 Figure 4 shows the inhibition of insulin-induced T-47D cell proliferation in 2% FBS by murine leptin as determined by crystal violet staining.

Figure 5 shows the inhibition of IGF-I-induced T-47D cell proliferation in 10% FBS by murine leptin as determined by MTT staining.

Figure 6 shows the inhibition of IGF-I-induced T-47D cell proliferation in 2% FBS by murine leptin as determined by crystal violet staining.

Figure 7 shows the inhibition of IGF-I-induced T-47D cell proliferation in 2% FBS by  
5 human leptin as determined by crystal violet staining.

Figure 8 shows the inhibition of insulin-induced MCF7 cell proliferation in serum-free  
by murine leptin as determined by crystal violet staining.

10 Figure 9 shows the inhibition of IGF-I-induced MCF7 cell proliferation in serum-free  
by murine leptin as determined by crystal violet staining.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention concerns the use of leptin as an inhibitor of tumor cell  
15 proliferation. Typically, cell lines of human origin, derived from various tumors, may be  
grown in culture in the presence of growth medium supplemented with fetal bovine serum at a  
concentration of about 10% by volume. Cell proliferation under these conditions is defined  
hereinafter as "basal cell proliferation". Growth of many tumor cell lines is significantly  
enhanced when various growth factors such as insulin, epidermal growth factor or insulin-like  
20 growth factor-I (IGF-I) are added to the above serum-supplemented culture medium.  
Inclusion of leptin in growth media at a range of concentrations from 3 to 600 nanomolar  
reduces both basal cell proliferation and growth-factor-dependent cell proliferation.

The results of the cell culture experiments given in the examples below indicate that  
leptin is useful for inhibiting the growth of various tumors. Hence, leptin may be useful for the  
25 treatment of various malignancies.

In a preferred embodiment of the present invention, leptin is used for the inhibition of  
breast cancer cell proliferation. When leptin is added to cultures of the human ductal breast  
carcinoma T-47D cells, (American Type Culture Collection, Rockville, MD; strain No. ATCC  
HTB 133), their extent of proliferation is reduced. Similarly, when leptin is added to cultures

of the human breast adenocarcinoma MCF7 cells, (American Type Culture Collection, Rockville, MD; strain No. ATCC HTB 22), their extent of proliferation is reduced. Leptin inhibits both the basal, the IGF-I-induced and the insulin-induced proliferation of T-47D and MCF7 cells. Hence, leptin may be useful specifically for the treatment of breast carcinomas.

5       The growth stimulatory effect of insulin and IGF-I on cells is mediated, at least in part, via tyrosine phosphorylation of IRS-1 and subsequent association of IRS-1 with GRB2, leading to a mitogenic response. The anti-mitogenic effect of leptin may result from its ability to reduce the basal, the insulin-induced and the IGF-I-induced tyrosine phosphorylation of IRS-1, leading to reduced binding of GRB2 to IRS-1. Furthermore, IRS-1 is a substrate of  
10 receptor kinases of other growth factors and cytokines, including IL-4 and IL-9 (Pernis et al., 1995; Yin et al., 1995; Yin et al., 1994). Therefore, leptin may inhibit the mitogenic responses of some or all of the aforementioned growth factors and cytokines, as well as other mitogens, thereby inhibiting the proliferation of a variety of tumor cells.

15      The present invention further relates to leptin derivatives and analogs, including leptin fusion proteins, leptin muteins, leptin receptor agonists, or active fragments or fractions thereof, and salts of all of same, and pharmaceutical compositions containing leptin, leptin fusion proteins, leptin muteins, leptin receptor agonists, active fractions thereof, or salts of all of same for the treatment of various cancers.

20      As used herein the term "muteins" refers to analogs of leptin, in which one or more of the amino acid residues are replaced by different amino acid residues, or are deleted, or one or more amino acid residues are added to the original sequence of leptin without changing considerably the activity of the resulting products as compared with wild type leptin or its active fragments or fractions. These muteins are prepared by known synthesis and/or by site-directed mutagenesis techniques, or any other known technique suitable therefor.

25      Any such mutein preferably has a sequence of amino acids sufficiently duplicative of that of leptin such as to have substantially similar activity to leptin or its active fragments or fractions. Thus, it can be determined whether any given mutein has substantially the same activity as leptin by means of routine experimentation comprising subjecting such a mutein, e.g., to a simple cell proliferation assay, as a mutein which blocks cell proliferation retains sufficient activity of leptin and therefore has at least one of the disclosed utilities of leptin and thus has substantially similar activity thereto.

In a preferred embodiment, any such mutein has at least 40% identity or homology with the sequence of one of the leptins. More preferably, it has at least 50%, at least 60%, at least 70%, at least 80% or, most preferably, at least 90% identity or homology thereto.

Muteins of leptin or its active fragments or fractions which can be used in accordance with the present invention, or nucleic acid coding therefor, include a finite set of substantially corresponding sequences as substitution peptides or polynucleotides which can be routinely obtained by one of ordinary skill in the art, without undue experimentation, based on the teachings and guidance presented herein. For a detailed description of protein chemistry and structure, see Schulz, G.E. et al., *Principles of Protein Structure*, Springer-Verlag, New York, 1978; and Creighton, T.E., *Proteins: Structure and Molecular Properties*, W.H. Freeman & Co., San Francisco, 1983, which are hereby incorporated by reference. For a presentation of nucleotide sequence substitutions, such as codon preferences, see Ausubel et al, *supra*, at §§ A.1.1-A.1.24, and Sambrook et al, *Current Protocols in Molecular Biology*, Interscience N.Y. §§6.3 and 6.4 (1987, 1992), at Appendices C and D.

Preferred changes for muteins in accordance with the present invention are what are known as "conservative" substitutions. Conservative amino acid substitutions of leptin polypeptides or proteins or its active fragments or fractions may include synonymous amino acids within a group which have sufficiently similar physicochemical properties that substitution between members of the group will preserve the biological function of the molecule, Grantham, *Science*, Vol. 185, pp. 862-864 (1974). It is clear that insertions and deletions of amino acids may also be made in the above-defined sequences without altering their function, particularly if the insertions or deletions only involve a few amino acids, e.g., under thirty, and preferably under ten, and do not remove or displace amino acids which are critical to a functional conformation, e.g., cysteine residues, Anfinsen, "Principles That Govern The Folding of Protein Chains", *Science*, Vol. 181, pp. 223-230 (1973). Proteins and muteins produced by such deletions and/or insertions come within the purview of the present invention.

Preferably, the synonymous amino acid groups are those defined in Table I. More preferably, the synonymous amino acid groups are those defined in Table II; and most preferably the synonymous amino acid groups are those defined in Table III.

**TABLE I Preferred Groups of Synonymous Amino Acids**

| Amino Acid | Synonymous Group                  |
|------------|-----------------------------------|
| Ser        | Ser, Thr, Gly, Asn                |
| Arg        | Arg, Gln, Lys, Glu, His           |
| 5 Leu      | Ile, Phe, Tyr, Met, Val, Leu      |
| Pro        | Gly, Ala, Thr, Pro                |
| Thr        | Pro, Ser, Ala, Gly, His, Gln, Thr |
| Ala        | Gly, Thr, Pro, Ala                |
| Val        | Met, Tyr, Phe, Ile, Leu, Val      |
| 10 Gly     | Ala, Thr, Pro, Ser, Gly           |
| Ile        | Met, Tyr, Phe, Val, Leu, Ile      |
| Phe        | Trp, Met, Tyr, Ile, Val, Leu, Phe |
| Tyr        | Trp, Met, Phe, Ile, Val, Leu, Tyr |
| Cys        | Ser, Thr, Cys                     |
| 15 His     | Glu, Lys, Gln, Thr, Arg, His      |
| Gln        | Glu, Lys, Asn, His, Thr, Arg, Gln |
| Asn        | Gln, Asp, Ser, Asn                |
| Lys        | Glu, Gln, His, Arg, Lys           |
| Asp        | Glu, Asn, Asp                     |
| 20 Glu     | Asp, Lys, Asn, Gln, His, Arg, Glu |
| Met        | Phe, Ile, Val, Leu, Met           |
| Trp        | Trp                               |

**TABLE II** More Preferred Groups of Synonymous Amino Acids

|    | Amino Acid | Synonymous Group        |
|----|------------|-------------------------|
| 5  | Ser        | Ser                     |
|    | Arg        | His, Lys, Arg           |
|    | Leu        | Leu, Ile, Phe, Met      |
|    | Pro        | Ala, Pro                |
|    | Thr        | Thr                     |
|    | Ala        | Pro, Ala                |
| 10 | Val        | Val, Met, Ile           |
|    | Gly        | Gly                     |
|    | Ile        | Ile, Met, Phe, Val, Leu |
|    | Phe        | Met, Tyr, Ile, Leu, Phe |
|    | Tyr        | Phe, Tyr                |
|    | Cys        | Cys, Ser                |
| 15 | His        | His, Gln, Arg           |
|    | Gln        | Glu, Gln, His           |
|    | Asn        | Asp, Asn                |
|    | Lys        | Lys, Arg                |
|    | Asp        | Asp, Asn                |
|    | Glu        | Glu, Gln                |
| 20 | Met        | Met, Phe, Ile, Val, Leu |
|    | Trp        | Trp                     |

**TABLE III** Most Preferred Groups of Synonymous Amino Acids

|    | Amino Acid | Synonymous Group |
|----|------------|------------------|
|    | Ser        | Ser              |
|    | Arg        | Arg              |
| 5  | Leu        | Leu, Ile, Met    |
|    | Pro        | Pro              |
|    | Thr        | Thr              |
|    | Ala        | Ala              |
|    | Val        | Val              |
| 10 | Gly        | Gly              |
|    | Ile        | Ile, Met, Leu    |
|    | Phe        | Phe              |
|    | Tyr        | Tyr              |
|    | Cys        | Cys, Ser         |
| 15 | His        | His              |
|    | Gln        | Gln              |
|    | Asn        | Asn              |
|    | Lys        | Lys              |
|    | Asp        | Asp              |
| 20 | Glu        | Glu              |
|    | Met        | Met, Ile, Leu    |
|    | Trp        | Met              |

Examples of production of amino acid substitutions in proteins which can be  
25 used for obtaining muteins of leptin or its active fractions for use in the present invention include any known method steps, such as presented in US patents RE 33,653, 4,959,314, 4,588,585 and 4,737,462, to Mark et al; 5,116,943 to Koths et al., 4,965,195 to Namen et al; 4,879,111 to Chong et al; and 5,017,691 to Lee et al; and lysine substituted proteins presented in US patent No. 4,904,584 (Shaw et al).

30 In another preferred embodiment of the present invention, any mutein of leptin or its active fractions for use in the present invention has an amino acid sequence essentially

corresponding to that of leptin. The term "essentially corresponding to" is intended to comprehend proteins with minor changes to the sequence of the natural protein which do not affect the basic characteristics of the natural proteins, particularly insofar as its ability to inhibit cell proliferation is concerned. The type of changes which are generally considered to fall within the "essentially corresponding to" language are those which would result from conventional mutagenesis techniques of the DNA encoding leptin, resulting in a few minor modifications, and screening for the desired activity in the manner discussed above.

Muteins in accordance with the present invention include proteins encoded by a nucleic acid, such as DNA or RNA, which hybridizes to DNA or RNA which encodes leptin in accordance with the present invention, under stringent conditions. Such nucleic acid would be a prime candidate to determine whether it encodes a polypeptide which retains the functional activity of leptin of the present invention. The term "stringent conditions" refers to hybridization and subsequent washing conditions which those of ordinary skill in the art conventionally refer to as "stringent". See Ausubel et al., Current Protocols in Molecular Biology, supra, Interscience, NY, §§6.3 and 6.4 (1987, 1992), and Sambrook et al., supra. Without limitation, examples of stringent conditions include washing conditions 12-20°C below the calculated Tm of the hybrid under study in, e.g., 2 x SSC and 0.5% SDS for 5 minutes, 2 x SSC and 0.1% SDS for 15 minutes; 0.1 x SSC and 0.5% SDS at 37°C for 30-60 minutes and then a 0.1 x SSC and 0.5% SDS at 68°C for 30-60 minutes. Those of ordinary skill in this art understand that stringency conditions also depend on the length of the DNA sequences, oligonucleotide probes (such as 10-40 bases) or mixed oligonucleotide probes. If mixed probes are used, it is preferable to use tetramethyl ammonium chloride (TMAC) instead of SSC. See Ausubel, supra.

The term "leptin fusion proteins" or simply "fused protein" refers to a polypeptide comprising leptin or its active fractions or a mutein thereof, fused with another protein which, e.g., has an extended residence time in body fluids. Leptin or its active fractions may thus be fused to another protein, polypeptide or the like.

The term "salts" herein refers to both salts of carboxyl groups and to acid addition salts of amino groups of leptin, its active fractions, muteins, or leptin fusion proteins thereof.

Salts of a carboxyl group may be formed by means known in the art and include inorganic salts, for example, sodium, calcium, ammonium, ferric or zinc salts, and the like, and salts with organic bases as those formed, for example, with amines, such as triethanolamine, arginine or lysine, piperidine, procaine and the like. Acid addition salts include, for example, 5 salts with mineral acids such as, for example, hydrochloric acid or sulfuric acid, and salts with organic acids such as, for example, acetic acid or oxalic acid. Of course, any such salts must have substantially similar activity to leptin or its active fractions.

"Functional derivatives" as used herein cover derivatives of leptin or its active fragments or fractions and its muteins and leptin fusion proteins, which may be prepared from 10 the functional groups which occur as side chains on the residues or the N- or C-terminal groups, by means known in the art, and are included in the invention as long as they remain pharmaceutically acceptable, i.e. they do not destroy the activity of the protein which is substantially similar to the activity of leptin, and do not confer toxic properties on compositions containing it. These derivatives may, for example, include polyethylene glycol 15 side-chains which may mask antigenic sites and extend the residence of leptin or its active fractions in body fluids. Other derivatives include aliphatic esters of the carboxyl groups, amides of the carboxyl groups by reaction with ammonia or with primary or secondary amines, N-acyl derivatives of free amino groups of the amino acid residues formed with acyl moieties (e.g. alkanoyl or carbocyclic aroyl groups) or O-acyl derivatives of free hydroxyl 20 groups (for example that of seryl or threonyl residues) formed with acyl moieties.

As "active fragments or fractions" of leptin, leptin muteins and leptin fusion proteins, the present invention covers any fragment or precursors of the polypeptide chain of leptin, or fused proteins containing any such fragment of leptin, alone or together with associated molecules or residues linked thereto, e.g., sugar or phosphate residues, or aggregates of any 25 of the above derivatives, provided said fraction has substantially similar activity to leptin.

The present invention further relates to use of natural and synthetic agonists of the leptin receptor, which are essentially similar to leptin in their ability to inhibit cell proliferation. Such agonists may be selected from a library of peptides, a library of peptide analogs or a random library of organic molecules. Selection is done by means known in the art, essentially 30 by the ability of the selected agonists to bind to the leptin receptor. For example, a library of random peptides may be prepared as prokaryotic expression plasmids carrying a DNA coding

for a random peptide, fused to a carrier protein. Another example is a phage display system in which the expression system is a phage containing DNA coding for a random peptide and incorporated into one of the external phage proteins. Phages coding for fused peptide agonists or antagonists are isolated from the phage library by e.g., panning over surfaces coated with 5 the leptin receptor. Bound phages are isolated and then amplified in bacteria. Several rounds of panning - amplification are usually required in order to obtain phages expressing fused peptides that have high affinity for the leptin receptor. The isolated phage is then amplified and the sequence of DNA coding for the peptide is determined. Alternatively, random peptide libraries or libraries of other molecules are prepared by solid phase synthesis on polymeric 10 beads by means known in the art. Beads carrying a peptide or other molecule having affinity for the leptin receptor are selected from the library, e.g., by binding of labeled leptin receptor, e.g., fluorescently labeled leptin receptor. Positive beads are then picked up and the structure of the peptide or other molecule present on the bead is determined. If the bead carries a peptide, the peptide sequence is determined by protein sequence analysis. If the bead is a 15 representative of a random library of organic molecules then the molecule is cleaved from the bead and its structure is determined by means known in the art, such as mass spectrometry, nuclear magnetic resonance and the like. Candidate peptides identified by their affinity for the leptin receptor are then further selected by their ability to inhibit cell proliferation in the aforementioned manner.

20 Accordingly, leptin, its active fractions, leptin muteins, leptin fusion proteins, leptin receptor agonists and their salts, functional derivatives, and active fragments or fractions thereof are indicated for the treatment of various malignancies, preferably for growth factor-dependent tumors and more preferably for breast carcinomas.

The present invention further relates to use of pharmaceutical compositions 25 comprising a pharmaceutically acceptable carrier and leptin of the invention, or its active muteins, fused proteins, leptin receptor agonists and their salts, functional derivatives or active fractions thereof.

The pharmaceutical compositions of the invention are prepared for administration by mixing leptin or its derivatives, or leptin receptor agonists with physiologically acceptable 30 carriers, and/or stabilizers and/or excipients, and prepared in dosage form, e.g., by lyophilization in dosage vials. The method of administration can be via any of the accepted

modes of administration for similar agents and will depend on the condition to be treated, e.g., intravenously, intramuscularly, subcutaneously, by local injection or topical application, or continuously by infusion, etc. The amount of active compound to be administered will depend on the route of administration, the disease to be treated and the condition of the patient. Local  
5 injection, for instance, will require a lower amount of the protein on a body weight basis than will intravenous infusion. Typical active amounts of leptin to be injected are 0.1-1000 microgram/kg body weight and preferably 1 to 10 micrograms/kg. Active amounts of leptin derivatives and leptin receptor agonists may be essentially the same as those of leptin on a molar basis.

10 Leptin may be administered to cancer patients, e.g., by injection, either alone or in combination with other therapeutic agents or in combination with other therapeutic approaches.

The invention will now be illustrated by the following non-limiting examples:

15

**EXAMPLE 1: Determination of cell proliferation by MTT staining**

Reagents:

MTT stock: (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide 5 mg/ml in phosphate-buffered saline. Stored at -20°C until used.  
20 Solvent: Conc. HCl (450 microliters) in 2-propanol (100 ml).

Procedure:

Grow cells in 96 well plates in the presence of various growth stimulants and growth inhibitors. At a desired time add MTT stock (10 microliters) to each well. Incubate 2.5-3 hours at 37°C. Aspirate supernatant with vacuum using a fine gauge needle. Add Solvent (100 microliters) and read the absorbance with a microplate reader using a 570 nM filter with background subtraction at 630 nm.  
25

**EXAMPLE 2: Determination of cell proliferation by crystal violet staining****Procedure:**

Grow cells in 96 well plates in the presence of various growth stimulants and growth inhibitors. At the desired time add 12.5% glutaraldehyde (40 microliters) to each well.

5 Incubate 30 minutes at room temperature. The microplate was then washed with water, dried and aq. crystal violet (0.1% , 0.1 ml) was added to each well. The microplate was further incubated for 30 min., washed with water and read at 540 nm with background subtraction at 630 nm.

**10 EXAMPLE 3: Determination of the insulin-dependent T-47D cell proliferation**

Human T-47D cells (American Type Culture Collection, Rockville, MD; strain No. ATCC HTB 133), were seeded into 96 well plates at  $3 \times 10^5$  cells/ml in DMEM and 10% fetal bovine serum (FBS), 0.1 ml per well. Human insulin was added to different wells at increasing concentrations, the plates were incubated for 72 hours and the number of cells was then determined by staining with MTT (Figure 1). The results are average of 8 replicates. Based on the extent of cell proliferation as shown in Figure 1, a concentration of 50 nM insulin was used for further studies.

**EXAMPLE 4: Determination of the IGF-I-dependent T-47D cell proliferation**

20 Human T-47D cells were seeded into 96 well plates at  $3 \times 10^5$  cells/ml in DMEM and 10% FBS, 0.1 ml per well. Human IGF-I was added to different wells at increasing concentrations, the plates were incubated for 3 days and the number of cells was then determined by MTT staining (Figure 2). The results are average of 8 replicates. Based on the extent of cell proliferation as shown in Figure 2, a concentration of 50 ng/ml IGF-I was used for further studies.

**EXAMPLE 5: Inhibition of insulin-induced T-47D cell proliferation by leptin**

T-47D cells ( $3 \times 10^5$  cells/ml) in DMEM supplemented with 10% FBS were seeded into 96 well plates (0.1 ml per well). Cells were treated with insulin (50 nM), with or without the

indicated concentrations of murine leptin. The Plates were incubated at 37°C in 5% CO<sub>2</sub> for 48 hours. The cells were then stained with MTT. The data are mean ± standard error (SE, n=8). The results show that leptin inhibited significantly the insulin-induced cell proliferation (**Figure 3**).

5 T-47D cells (3x10<sup>5</sup> cells/ml) in DMEM supplemented with 10% FBS were seeded into 96 well plates (0.1 ml per well). After one day the medium was replaced by DMEM supplemented with 2% FBS and after one day the cells were treated with insulin (50 nM), with or without the indicated concentrations of murine leptin in DMEM-2% FBS. The Plates were incubated at 37°C in 5% CO<sub>2</sub> for 48 hours. The cells were then stained with crystal  
10 violet. The data are mean ± SE, (n=8). The results show that leptin inhibited significantly the insulin-induced cell proliferation (**Figure 4**).

#### EXAMPLE 6: Inhibition of IGF-I-induced T-47D cell proliferation by leptin

15 T-47D cells (3x10<sup>5</sup> cells/ml) in DMEM supplemented with 10% FBS were seeded into 96 well plates (0.1 ml per well). Cells were treated with IGF-I (50 ng/ml), with or without the indicated concentrations of murine leptin. The Plates were incubated at 37°C in 5% CO<sub>2</sub> for 48 hours. The cells were then stained with MTT. The data are mean ± standard error (SE, n=8). The results show that leptin inhibited significantly the IGF-I-induced cell proliferation  
20 (**Figure 5**).

20 T-47D cells (3x10<sup>5</sup> cells/ml) in DMEM supplemented with 10% FBS were seeded into 96 well plates (0.1 ml per well). After one day the medium was replaced by DMEM supplemented with 2% FBS and after one day the cells were treated with IGF-I (50 ng/ml), with or without the indicated concentrations of murine leptin in DMEM-2% FBS. The Plates were incubated at 37°C in 5% CO<sub>2</sub> for 48 hours. The cells were then stained with crystal  
25 violet. The data are mean ± standard error (SE, n=8). The results show that leptin inhibited significantly the IGF-I-induced cell proliferation (**Figure 6**).

30 T-47D cells (3x10<sup>5</sup> cells/ml) in DMEM supplemented with 10% FBS were seeded into 96 well plates (0.1 ml per well). After one day the medium was replaced by DMEM supplemented with 2% FBS and after one day the cells were treated with IGF-I (50 ng/ml), with or without the indicated concentrations of human leptin in DMEM-2% FBS. The Plates were incubated at 37°C in 5% CO<sub>2</sub> for 48 hours. The cells were then stained with crystal

violet. The data are mean  $\pm$  standard error (SE, n=8). The results show that leptin inhibited significantly the IGF-I-induced cell proliferation (**Figure 7**).

**EXAMPLE 8: Inhibition of insulin-induced MCF7 cell proliferation by leptin**

5 Human breast adenocarcinoma MCF7 cells ( $3 \times 10^4$  cells/ml, American Type Culture Collection, Rockville, MD; strain No. ATCC HTB 22) supplemented with 6% FBS were seeded into 96 well plates (0.1 ml per well). After one day the medium was replaced by serum-free DMEM and after one day the cells were treated with insulin (50 nM) with or without the indicated concentrations of murine leptin in a serum-free medium. The Plates  
10 were incubated at 37°C in 5% CO<sub>2</sub> for 48 hours. The cells were then stained with crystal violet. The data are mean  $\pm$  SE, (n=8). The results show that leptin inhibited significantly the insulin-induced cell proliferation (**Figure 8**).

**EXAMPLE 9: Inhibition of IGF-I-induced MCF7 cell proliferation by leptin**

15 Human breast adenocarcinoma MCF7 cells in DMEM supplemented with 10% FBS were seeded into 96 well plates ( $3 \times 10^4$  cells/ml, 0.1 ml per well). After one day the medium was replaced by a serum-free medium. After one day the cells were treated with IGF-I (50 ng/ml) with or without the indicated concentrations of murine leptin in a serum-free medium. The Plates were incubated at 37°C in 5% CO<sub>2</sub> for 96 hours. The cells were then stained with crystal violet. The data are mean  $\pm$  SE, (n=8). The results show that leptin inhibited significantly the insulin-induced cell proliferation (**Figure 9**).

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CLAIMS

1. The use of an active agent selected from the group consisting of leptin, leptin fusion proteins, leptin muteins, leptin receptor agonists, active fragments or fractions of any one thereof, active analogs or derivatives of any one thereof, salts of any one thereof, and mixtures of any thereof, as an inhibitor of tumor cell proliferation.
2. The use of an active agent according to claim 1 as an inhibitor of cell proliferation for the treatment of malignancies in mammals.
3. The use of an active agent according to claim 1 or claim 2 as an inhibitor of growth-factor dependent tumors.
4. The use of an active agent according to any one of claims 1-3, as an inhibitor of human breast carcinoma cell proliferation.
5. The use of an active agent according to claim 4 for the treatment of human breast carcinomas.
6. The use of an active agent according to claim 1 or claim 3 as an inhibitor of the growth stimulatory effect of insulin on tumor cells, as mediated, at least partially, by the insulin receptor substrate-1 (IRS-1)/growth-factor receptor-associated binding protein-2 (GRB2) pathway.
7. The use of an active agent according to claim 1 or claim 3 as an inhibitor of the mitogenic responses in tumor cells to one or more receptor kinases, growth factors and cytokines of the group consisting of IGF-1, IL-4 and IL-9, for all of which IRS-1 is a substrate, for the treatment of tumors.
8. The use of an active agent according to any one of claims 1-7 as an inhibitor of basal and insulin-induced tumor cell proliferation for the treatment of human breast cancers.

9. The use of an active agent according to any one of claims 1-8, wherein said active ingredient is leptin, and said leptin is used as said inhibitor or for said treatment.

5 10. An active agent selected from the group consisting of leptin, leptin fusion proteins, leptin muteins, leptin receptor agonists, active fragments or fractions of any one thereof, active analogs or derivatives of any one thereof, salts of any one thereof, and mixtures of any thereof, for use in the preparation of a medicament for the inhibition of tumor cell proliferation.

10 11. An active agent according to claim 10 for use in the preparation of a medicament for the treatment of malignancies in mammals.

12. An active agent according to claim 10 or claim 11 for use in the preparation of a  
15 medicament for the inhibition of growth-factor-dependent tumors.

13. An active agent according to any one of claims 10-12 for use in the preparation of a medicament for the inhibition of human breast carcinoma cell proliferation.

20 14. An active agent according to claim 13 for use in the preparation of a medicament for the treatment of human breast carcinomas.

15. An active agent according to claim 10 or claim 12 for use in the preparation of a medicament for the inhibition of the growth stimulatory effect of insulin on tumor cells, as  
25 mediated, at least partially, by the IRS-1/GRB2 pathway.

16. An active agent according to claim 10 or claim 12 for use in the preparation of a medicament for the inhibition of the mitogenic responses in tumor cells to one or more receptor kinases, growth factors and cytokines of the group consisting of IGF-1, IL-4 and  
30 IL-9, for all of which IRS-1 is a substrate, for the treatment of tumors.

17. An active agent according to any one of claims 10-16, for use in the preparation of a medicament for the inhibition of basal and insulin-induced tumor cell proliferation, for the treatment of human breast cancers.

5 18. An active agent according to any one of claims 10-17, wherein said active agent is leptin, and said leptin is used for the preparation of said medicament.

19. A pharmaceutical composition comprising as active ingredient an active agent according to claim 1 or claim 10 and a pharmaceutically acceptable carrier, diluent or 10 excipient, for the inhibition of tumor cell proliferation.

20. A pharmaceutical composition according to claim 19 for the treatment of malignancies in mammals.

15 21. A pharmaceutical composition according to claim 19 or 20 for the inhibition of growth-factor-dependent tumors.

22. A pharmaceutical composition according to any one of claims 19-21 for the inhibition of human breast carcinoma cell proliferation and thereby for the treatment of human breast 20 carcinoma.

23. A pharmaceutical composition according to claim 19 or 21 for the inhibition of the growth stimulatory effect of insulin on tumor cells, as mediated, at least partially, by the IRS-1/GRB2 pathway.

25 24. A pharmaceutical composition according to claim 19 or 21 for the inhibition of mitogenic responses in tumor cells to one or more receptor kinases, growth factors and cytokines of the group consisting of IGF-1, IL-4 and IL-9, for all of which IRS-1 is a substrate, and thereby for the treatment of tumors.

25. A pharmaceutical composition according to any one of claims 19-24 for the inhibition of basal and insulin-induced tumor cell proliferation and thereby for the treatment of human breast cancers.

5 26. A pharmaceutical composition according to any one of claims 19-25, wherein said active ingredient is leptin.

10 27. A method for treating tumors in mammals or for inhibiting tumor cell proliferation in mammals comprising administering to a patient a pharmaceutical composition according to any one of claims 19-26 in a suitable dosage form and by a suitable route of administration.

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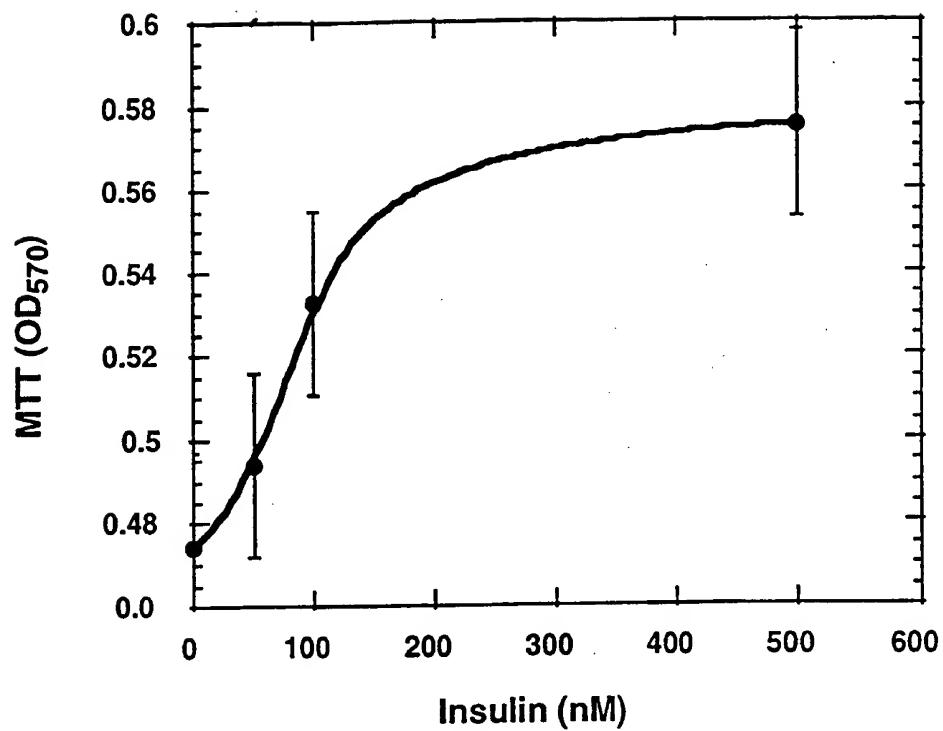
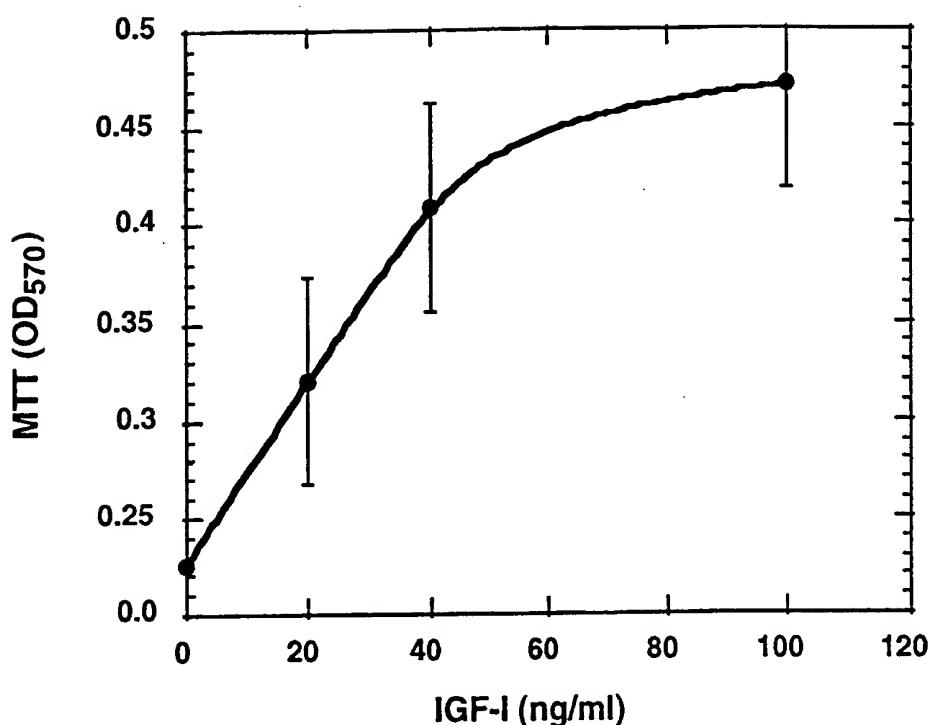


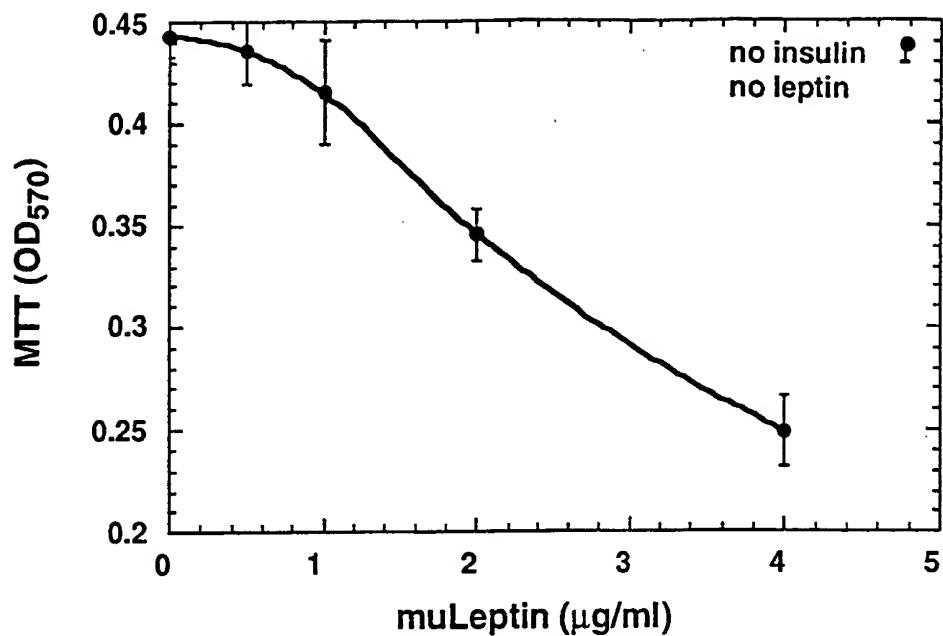
Figure 1

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**Figure 2**

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**Figure 3**

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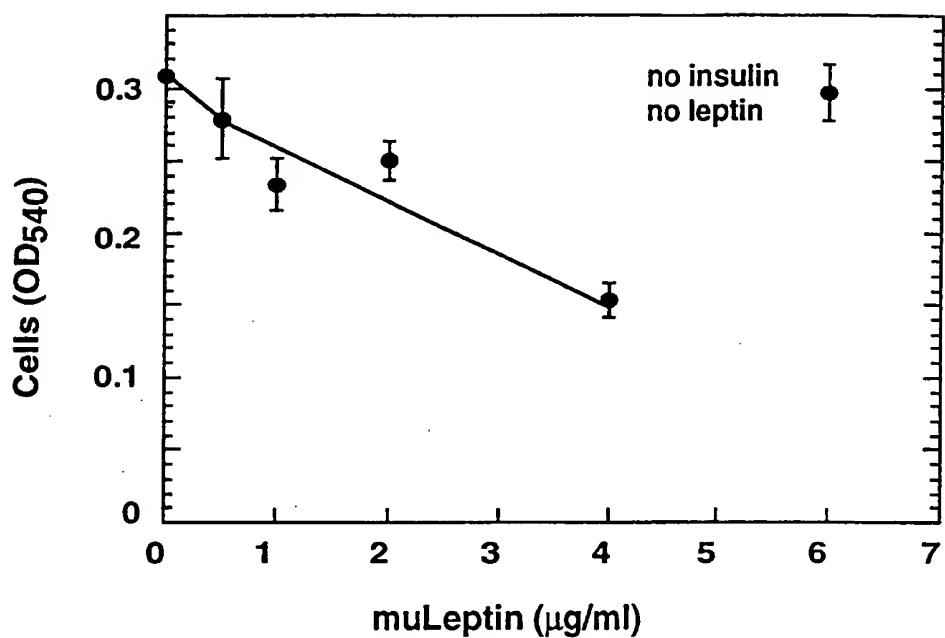
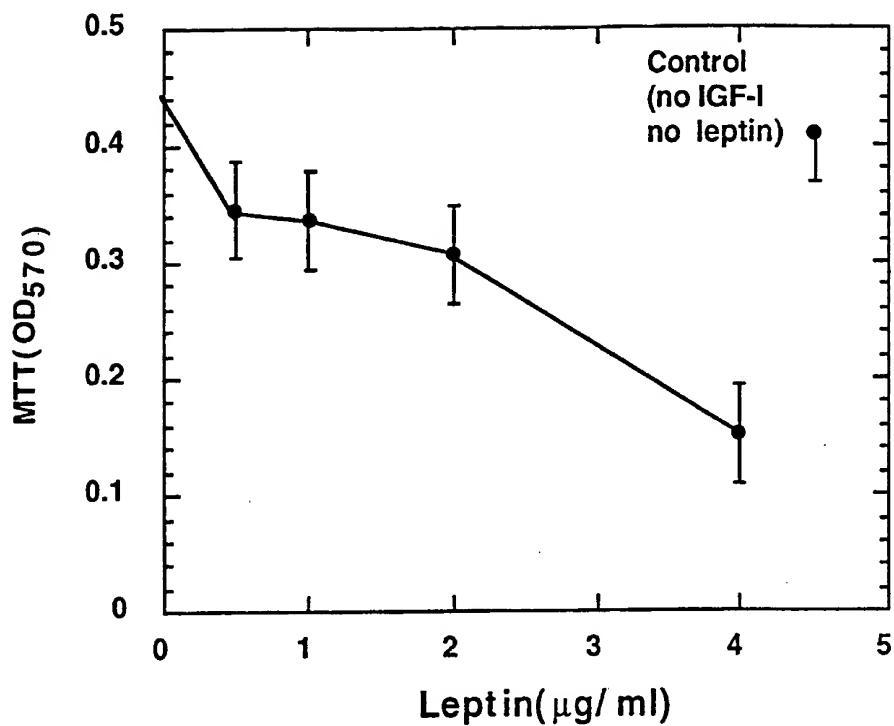


Figure 4

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**Figure 5**

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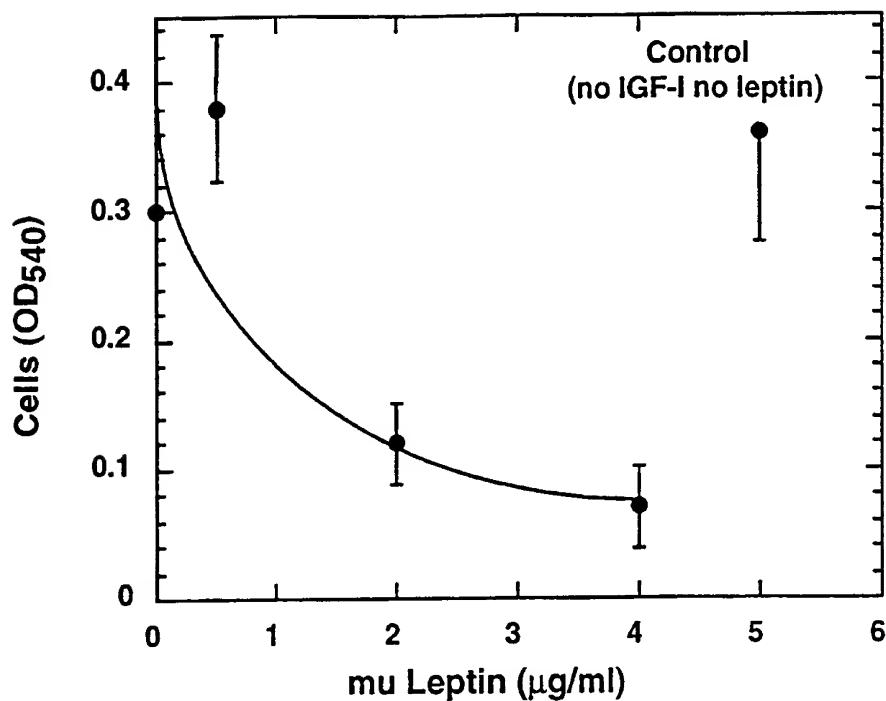


Figure 6

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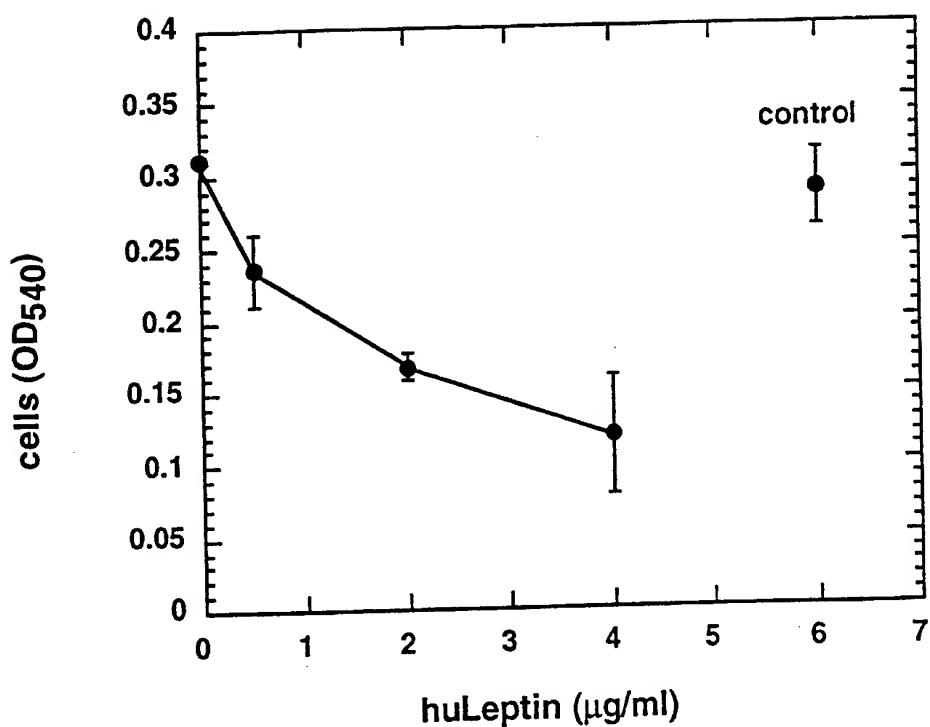
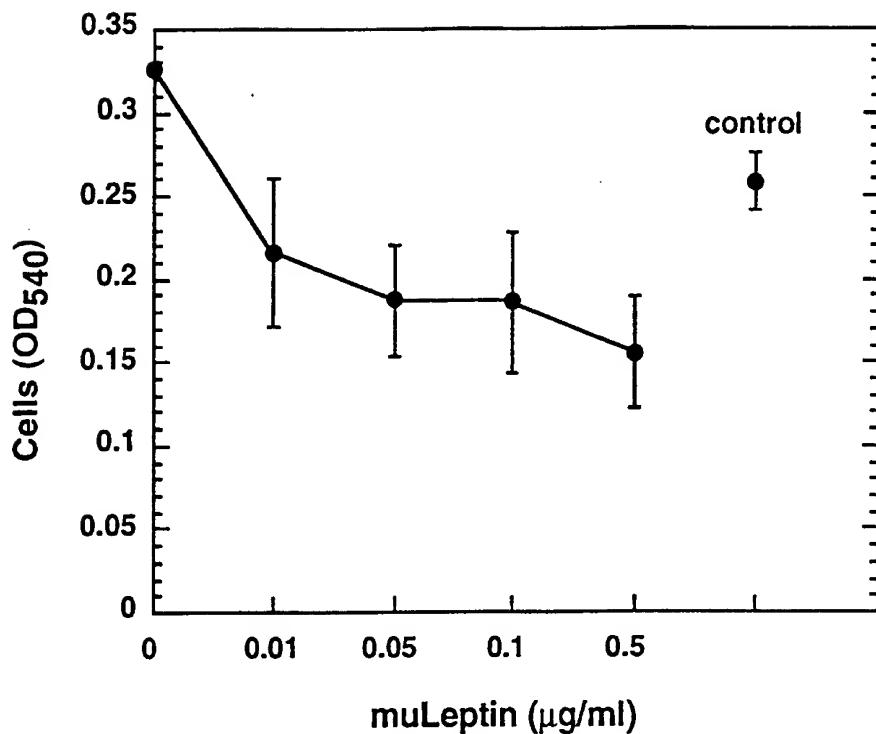


Figure 7

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**Figure 8**

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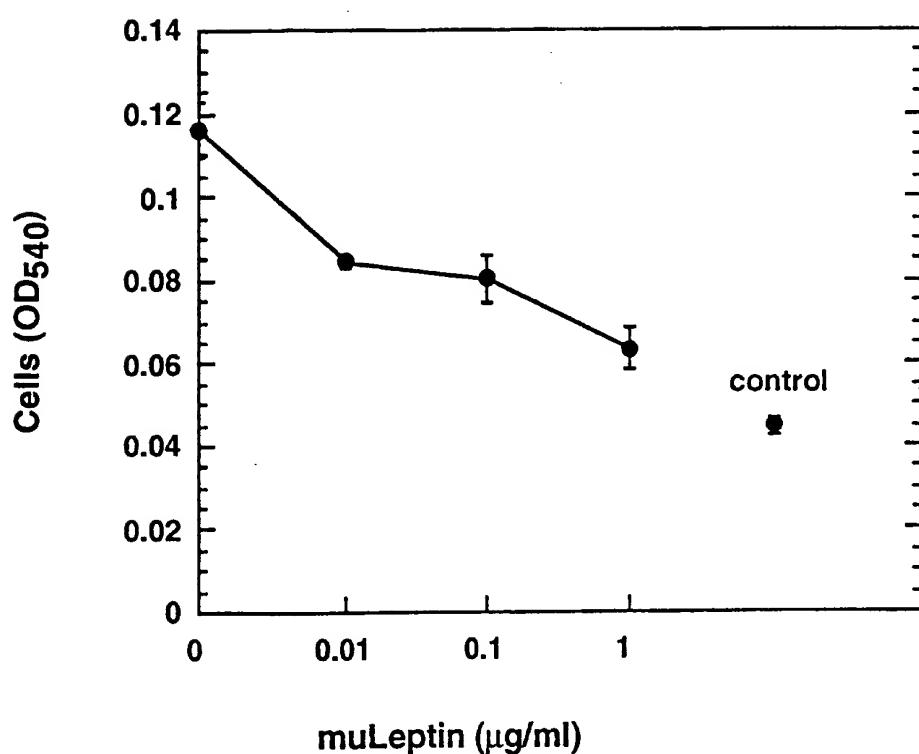


Figure 9

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/IL 98/00196

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC 6 A61K38/33

According to International Patent Classification(IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
|------------|--|-----------------------|
| P, X       | <p>RUBINSTEIN M., BARKAN D. ET AL.: "Leptin inhibits growth factor induced cell proliferation"<br/>         FIFTH ANN. CONFERENCE OF THE INT. CYTOKINE SOC. LAKE TAHOE,<br/>         vol. cytokine 9, no. 11, 9 - 13 November<br/>         1997, NEVADA, USA,<br/>         page 953 XP002074549<br/>         *cf. abstract*</p> <p>---</p> <p style="text-align: center;">-/--</p> | 1-27                  |

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

° Special categories of cited documents :

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- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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| Date of the actual completion of the international search  | Date of mailing of the international search report |
| 10 September 1998  | 18/09/1998   |
| Name and mailing address of the ISA<br><br>European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl.<br>Fax: (+31-70) 340-3016 | Authorized officer<br><br>Stoltner, A              |

## INTERNATIONAL SEARCH REPORT

International Application No

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document, with indication, where appropriate, of the relevant passages  | Relevant to claim No. |
|----------|---|-----------------------|
| Y        | ZACHOW R.J. , MAGOFFIN D.A.: "Direct intraovarian effects of leptin: Impairment of the synergistic action of insulin-like growth factor I on follicle-stimulating hormone dependent estradiol-17.beta production by rat ovarian granulosa cells" ENDOCRINOLOGY, vol. 138, no. 2, February 1997, pages 847-850, XP002074550<br>*cf. abstract*<br>----- | 1-27                  |
| P,Y      | WO 97 27286 A (PROGENITOR, INC.) 31 July 1997<br>*cf. page 5, lines 23-28, page 6, lines 21-37, page 9, lines 5-10, page 33, lines 25-31*<br>-----  | 1-27                  |

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International Application No  
PCT/IL 98/00196

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|--|------------------|-------------------------|------------------|
| WO 9727286 A                           | 31-07-1997       | AU 1831197 A            | 20-08-1997       |